RALSTONIA AHL-ACYLASE GENE

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention pertains to the field of molecular biology. In particular, the invention relates to an N-acyl homoserine lactone acylase gene from *Ralstonia sp.* XJ12B.

Description of the Background Art

[0002] N-acyl homoserine lactones (AHLs), also known as autoinducers, are widely used quorum sensing signal molecules in many Gram-negative bacteria. These compounds regulate certain classes of target genes in bacteria, such as virulence genes or biofilm differentiation genes. Generally, quorum sensing molecules are highly conserved and share an identical homoserine lactone moiety. The length and structure of their acyl side chains are different, however. Although the target genes regulated by AHLs in different bacteria species are varied, basic mechanisms of AHL biosynthesis and gene regulation are conserved among different bacterial species.

[0003] The general feature of AHL-mediated gene regulation is that it is cell population dependent (quorum sensing). Bacteria secrete AHLs into the environment; extracellular concentration of AHLs increases as bacterial cell populations grow. When AHL accumulates to a threshold extracellular concentration, the expression of certain sets of target genes are triggered in the bacteria.

[0004] Bacteria using these signals release, detect and respond to the accumulation of AHL signal molecules for synchronizing expression of a particular sets of genes and coordinating cellular activities within the bacterial cell population. AHLs are involved in regulation of a range of biological functions, including bioluminescence in *Vibrio* species (13, 4), Ti plasmid

conjugal transfer in *Agrobacterium tumefaciens* (31), induction of virulence genes in *Burkhholderia cepacia*, *Erwinia carotovora*, *Erw. chrysanthemi*, *Erw. stewartii*, *Pseudomonas aeruginosa*, and *Xenorhabdus nematophilus* (3, 6, 12, 17, 19, 22, 23, 24, 26), regulation of antibiotic production in *P. aureofaciens* and *Erw. carotovora* (6, 26), swarming motility in *Serratia liquifaciens* (14) and biofilm formation in *P. fluorescens* and *P. aeruginosa* (1, 8). In many other bacterial species the relevant biological functions controlled by AHLs remain to be investigated (2, 5, 11).

[0005] A number of plant, animal and human bacterial pathogens use AHL quorum-sensing signals to regulate expression of pathogenic genes and aid in the formation of biofilms. Therefore, AHL quorum-sensing signal molecules are group of molecular targets for genetic and chemical manipulations since disruption of these signaling mechanisms can prevent or reduce the ability of these bacteria to infect plant and animal tissues or to form biofilms.

[0006] The gene encoding an AHL-inactivation enzyme (AiiA) from a Gram-positive bacterium (Bacillus strain 240B1) has been cloned (9). AiiA (also known as AHL-lactonase) inactivates AHL activity by hydrolyzing the lactone bond of AHLs (10). Expression of *aiiA* in transformed *Erw. carotovora* (a pathogenic strain which causes soft rot disease in many plants) significantly reduces the release of AHL, decreases extracellular pectrolytic enzyme activities, and attenuates pathogenicity on potato, eggplant, Chinese cabbage, carrot, celery, cauliflower, and tobacco (9). Transgenic plants expressing AHL-lactonase showed a significantly enhanced resistance to *Erw. carotovora* infection and delayed development of soft rot symptoms (10). AHL-inactivation mechanisms appear to be widely distributed. For example, a bacterial isolate of *Variovorax paradoxus* was reported to use AHL molecules as its energy and nitrogen sources, indicating the possible presence of AHL-degrading enzymes (18).

[0007] Further methods to counteract AHL-mediated plant, animal and human disease and plant pathogen virulence by interfering with bacterial intercellular communication would be highly desirable.

SUMMARY OF THE INVENTION

[0008] Accordingly, in this study, the cloning and characterization of a gene encoding an AHL-acylase from a bacterial isolate *Ralstonia* sp. JX12B is reported.

[0009] In one embodiment, the invention provides a composition of matter which comprises a nucleic acid according to SEQ ID NO: 1. In another embodiment, the invention provides a composition of matter which comprises a nucleic acid selected from the group consisting of nucleotides 1234-3618 of SEQ ID NO: 1, a fragment thereof and a substantially homologous variant thereof.

[00010] In yet a further embodiment, the invention provides a nucleic acid according to claim 2 which comprises nucleotides 1234-3618 of SEQ ID NO: 1.

[00011] In yet a further embodiment, the invention provides a composition of matter which comprises a peptidic sequence selected from the group consisting of a peptidic sequence according to SEQ ID NO: 2, a fragment thereof and a substantially homologous variant thereof.

[00012] In yet a further embodiment, the invention provides a composition of matter which comprises a peptidic sequence encoded by a nucleic acid selected from the group consisting of nucleotides 1234-3618 of SEQ ID NO: 1, a fragment thereof and a substantially homologous variant thereof.

[00013] In yet a further embodiment, the invention provides a composition of matter which comprises a peptidic sequence selected from the group consisting of SEQ ID NO: 2, a fragment thereof, a subunit thereof and a substantially homologous variant thereof, such as a peptidic sequence according to SEQ ID NO: 2, a peptidic sequence comprising amino acids 36-217 233-794**[?]** of SEQ ID NO: 2 or a peptidic sequence comprising amino acids 233-794 of SEQ ID NO: 2.

[00014] In yet a further embodiment, the invention provides a composition of matter as described above which inactivates AHL.

[00015] In yet a further embodiment, the invention provides a method of modulating AHL signaling activity which comprises contacting said AHL with a composition of matter as described above.

[00016] In yet a further embodiment, the invention provides a transgenic plant or non-human mammal harboring a nucleic acid as described above.

[00017] In yet a further embodiment, the invention provides a method of controlling a bacterial disease in a mammal which comprises administering to said mammal a composition of matter as described above, wherein the expression of pathogenic genes of said bacteria are regulated by AHL signals.

[00018] In yet a further embodiment, the invention provides a method of controlling a bacterial disease in a plant which comprises administering to said plant a composition of matter as described above, wherein the expression of pathogenic genes of said bacteria are regulated by AHL signals.

BRIEF DESCRIPTION OF THE DRAWINGS

[00019] Figure 1 is a photograph showing AHL inactivation bioassay results for bacterial cultures and bacterial proteins from the indicated bacterial clones. Figure 1A shows the results of a bioassay with bacterial cultures of *E. coli* DH5α strains 13H10 (slice 1), 2B10 (slice 2), MUB3 (slice 3), MUC6 (slice 4), GST-QsbA (slice 5) and GST (slice 6), which contain plasmid clones or constructs p13H10, p2B10, pMUB3, pMUC6, pGST-QsbA, and pGST, respectively. Figure 1B shows results for bioassay of the indicated bacterial proteins GST-QsbA and GST.

[00020] Figure 2 is a graph showing the temperature and pH optimum profiles of AHL acylase.

[00021] Figure 3 is a graph showing the time course of OOHL inactivation by the purified AHL-acylase.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[00022] A bacterial isolate of *Ralstonia sp.* XJ12B from a biofilm sample in a water treatment system was found to enzymatically inactivate AHLs, bacterial quorum-sensing molecules, in a bioassay using *Agrobacterium tumefaciens* strain Nt1 (*traR; tra::lacZ749*) as an indicator for AHL activity. The gene encoding the protein exhibiting this enzyme activity for AHL inactivation (*qsbA*) was cloned from a bacterial strain isolated from the biofilm sample and found to encode a peptide of 794 amino acids.

[00023] Bacterial cultures and bacterial proteins were assayed for the ability to inactivate AHL using *Agrobacterium tumefaciens* indicator cells. *A tumefaciens* was cultured at 28°C in MM medium as described in Zhang et al. (31). The bacteria or protein to be assayed is first mixed with an AHL substrate, for example N-β-oxooctanoyl-L-homoserine lactone (OOHL), and the reaction (inactivation of the AHL) is allowed to proceed. If AHL inactivation activity is present in the sample (*i.e.* the AHL has been cleaved and inactivated), then the inactivated AHL products fail to trigger the expression of *lacZ* reporter gene which is under the control of a TraR-dependent promoter. The strain *A. Tumefaciens* NT1 hosting the *lacZ* reporter system therefore does not turn blue in the presence of substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). See Example 2 for details of the bioassay. Any AHL may be used in the assay, as desired. Of course, any suitable assay for cleavage of AHL, including traditional *in vitro* enzyme assays may be used to detect the AHL inactivation activity. Those of skill in the art are able to modify or devise assays to detect and/or quantitate AHL inactivation.

[00024] *Escherichia coli* strain DH5a was used as a host for DNA manipulation. Both *Ralstonia sp.* and *E. coli* were cultured in LB medium (tryptone, 10 g/L, yeast extract, 5 g/L, and NaCl, 10 g/L, pH 7.0) at 37°C. Appropriate antibiotics were added when necessary at the following concentrations: ampicillin, 100 µg/ml; tetracycline, 10 µg/ml; and kanamycin, 20 µg/ml.

[00025] The gene encoding the protein responsible for the detected AHL inactivation was isolated using a cosmid library of 1600 clones with the genomic DNA of *Ralstonia sp.* strain XJ12B, constructed in *E. coli*. *E. coli* transfectants were screened for the ability to inactivate AHL. One clone, pl3H10, was found to inactivate AHL. Cosmid DNA from pl3H10 was digested, fused into a cloning vector, ligated and transformed into *E. coli*. The *E. coli* clones again were assayed for AHL inactivating activity. One clone, containing a 4 kb insert, had AHL inactivation activity.

[00026] Plasmids were subsequently purified for sequencing. The 4 kb fragment from clone p2B10 was completely sequenced according to known methods using ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems). See Table I, below. The sequence contained an open reading frame of 2385 nucleotides which was the AHL inactivation gene, *qsbA*, encoding a predicted polypeptide of 794 amino acids (85,373 Daltons).

Table I. *QsbA* Gene (*Ralstonia* sp.) Nucleotide Sequence (SEQ ID NO: 1).

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gtttgggaaagtgggnagcgcgctgtgcag cgccccgcccctcagccgcgcagctcggcg cgcaccgaatgcgcgcgccggtgggcgccc 90
ggcggctggccggtgtggcgccggatcagg cgccggaaggcggacatgtcgtgataaccg cactgttcggcgattgccgtcaggctcagc 180
gtgctgacttccagcaggtggcaggcgcgc tccacgcgcagccggtgcagcaattgcagc ggcgaggtgcccagggtcttggtgaaatgc 270
cgcagcagcgtgcgctcgctggtcgaggcg gcggcggccagcttggccaggtcgaacggc tcgtgcaggtgctgctgcaggtagcgccgc 360
gcccgcagtaccacgctggtgcggatggcg ggcttgctgcgcagccagatggcggtggac tcaccgcgcgacgggtggtcgagcacggcc 450
tggccgagggtgcgtgccagccgggtgtcg gccaggcggccgaccaggcgctgcgtgagc gccacgccgtgctccatcgcgcgcgccgtc 540
agcacgttgccgctgctgacgatggcctgc tccgccaccaccttcagctgcgggtagttg ccgtgcagccagccggcgatcagccacgtc 630
accgtcaagcgccggccggcgggcagcgcg ccggccagcagcgccacgccggtgaaggac gaggccaccaagcctgccggcgtccaggta 720
gcgccggatggtggcgcgttcccactccag cagggccaggcgctgctccagcgtgctgat gtggtcgaaatgcaggggcgggacgaccag 810
cgcgtcgcccagcgcggcgtcgcccgccgg cagcggctggcagcggcaggccagggtctc ggcggcggcctgccagcgggccgggtcgcg 900
cgcgaccagccgccacccgaacaccgggct ggcggcatcggcacgcttgccggcatgcat ggaggcgagcgcattggccacgccgagggt 990
gtcggcgacggtcgccagcgtggagaggcc ggcgtcgggaaaggtcagcaggtcgatgtc ggcatccgcaaagtataggggaggcgggcg 1080
gaggcctcctgcgtggcgggattgacccca actctggcgggaatacctctttcctccggg cgggccccagtcgacgatacggcggtggct 1170
gcgcctgcgcgccgccgcaagactagagcg acacaagacaagaccgacaacaggagacaa cgcATGATGCAGGGATTCGCGCTGCGCGGC 1260
ACGCTCGCCATGGCCGCGCTCGCGGCGCTG GCCGGCTGCGCCAGTTCCACCGATGGCCGC TGGGGGTCGCTCAGCGACACCGGCCTGTCC 1350
GCCGAGATCCGCCGCACCGGCTTCGGCATT CCGCACATCCGCGCCAACGACTACGCCAGC CTCGGCTATGGCATGGCCTATGCCTACGCG 1440
CAGGACAACCTGTGCCTGCTGGCCGACCAG GTGGTCACCGTCAACGGCGAGCGCTCGAAG ACCTTCGGGCCCGAGGGCACCGTGACGGTC 1530
TCGTTCAAGCCGATCCCCAACCTGCAGTCG GACGCCTTCTTCAAGGGCATCTTCGACGAG GACGGCCTGCGCGCCGGTTATGCGCAGATG 1620
TCGCCCGAGGCGCGCGAGCTGCTGCGCGGC TACATCGCCGGCTTCAACCGCTATCTCAAG GACACGCCGCCCGCCAACTTCCCGGCCGCC 1710
TGCCGCAATGCCGCCTGGGTGCGTCCGCTC ACGCTGGGCGACATGATGCGCATGGGCGAA GAGAAGGCGATCCAGGCCAGCGCCGGCGCC 1800
ATGCTGGCGGGCATCGTCGCCGCGCAGCCG CCGGGCCGCACGCCGGTGGCCGAGCGCGAG ATTCCGCCGCAGGCCGTCGACACCGTGGCG 1890
CTGGACCGCGAACTGCAGCTGCGCGACATG CCGATCGGCTCCAACGGCTGGGCCTTCGGC GCTGACGCCACCGCCAACCGGCGCGGCGTG 1980
CTGCTCGGCAATCCGCACTTCCCGTGGACG ACCACCAACCGCTTCTACCAGGTCCACCTG ACGGTGCCCGGCAAGCTCGACGTGATGGGC 2070
GCCTCGATCGCGGCCTTCCCGGTGGTGAGC ATCGGCTTCAACAAGGACGTGGCGTGGACG CACACCGTCTCCACCGGCCGCCGCTTCACC 2160
TTGTTCGAACTGAAGCTGGCCGAAGGCGAC CCGACCACCTACCTGGTCGACGGCACGCCG CACAAGATGACCACCCGCACGGTCGCCTTC 2250
GACGTGAAGCTGCCGGACGGCCGCCTCGAG CGCCGCACGCACACCTTCTACGACACCATC TACGGCCCGGTGCTGTCGATGCCGAGCGGC 2340
GGCATGCCGTGGACCACGCAGAAGGCCTAC GCCCTGCGCGACGCCAACCGCAACAACACG CGCTCGGTCGACAGCTGGCTGCATATCGGG 2430
CAGGCCCGGGACGTGGCCGGCATCCGCCAG GCCATCGGCAACCTGGGCATTCCCTGGGTC AACACCATCGCCACCGACCGCAACGGCCGC 2520
GCGCTGTTCGCCGACGTGTCGACCACGCCG GACGTGCCGGCCGCGGAGCTCCAGCGCTGT GCCCCGTCGCCGCTGGCCGGCAAACTCTTC 2610
AAGGACGCGGGCCTGGTGCTGCTCGACGGC TCGCGCGGCACCTGCAACTGGCAGGTCGAT CCGGCTTCGCCGGTACCCGGGCTGGTGGCG 2700
CCCGCGCGCATGCCGGTGCTCGAGCGCGAC GACTACGTCGCCAACAGCAATGACAGCTCC TGGCTGACCAACCCCGCGCAAAAGCTGACC 2790
GGCTTCTCGCCGGTGATGGGCTCGGTCGAC GTACCGCAGCGGCTGCGCACGCGCATCGGC CTGATCGAGATCGGCCGCCGCCTGGCCGGC 2880
ACCGACGGACTGCCCGGCAACCGCATCGAT CTGCCGAACCTGCAGGCGATGATCTTCAGC AATGCCAACCTGGCGGGACAACTGGTGCTG 2970
GGCGACCTGCTCGCGGCATGCAAGGCCACG CCGGCCCCGGATGCCGACGTGCGCGACGGC TGCGCCGCCCTCGGCCAGTGGAACCGCACC 3060
AGCAACGCCGACGCCCGCGCCGCGCACCTG TTCCGCGAGTTCTGGATGCGCGCCAAGGAC ATCGCGCAGGTGCACGCCGTCGAGTTCGAC 3150
CCGGCCGACCCGGTCCACACGCCGCGCGGC CTGCGCATGAACGACGCGACGGTACGCACG GCGGTGTTCAAGGCGCTGAAGGAAGCCGTG 3240
GGCGCGGTGCGCAAGGCGGGCTTCGCGCTG GATGCGCCGCTGGGCACGGTACAGGCCGCG CACGCACCGGACGGCTCCATCGCCCTGCAC 3330
GGCGGCGAGGAATACGAAGGCGTGCTCAAC AAGCTGCAAACCCTGCCGATCGGGCCGAAG GGGCTGCCGGTGTATTTCGGCACCAGCTAC 3420
ATCCAGACCGTGACCTTCGACGACCAGGGC CCGGTCGCCGACGCCATCCTCACCTACGGC GAATCGACCGACCACGCCTCGCCGCACGCG 3510
TTCGACCAGATGCGTGCGTACTCGGGCAAG CACTGGAACCGGCTGCCGTTCTCCGAAGCG GCCATCGCGGCCGATCCGGCGCTGAAGGTG 3600
ATGCGGTTGTCGCAGTGAgggctgccggtg cctggaaaaacgccccgcttgtgcggggcg ttttttgccagtgtgaatggctcaatcgt 3690
gttggaaaccgcatccggacatgactgtat tgtgactctgcctgtgtccgtgt 3743
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The predicted open reading frame of the *qsbA* gene is shown in upper case letters with the start codon and stop codon in bold. A putative ribosome binding site (AGGAGA) is underlined.

[00027] Sequence analysis of this peptide indicated that *QsbA* did not have any significant homology with the known AHL-lactonase quorum-sensing molecule inactivator encoded by the *aiiA* gene from *Bacillus sp.* 240B1, however the deduced peptide sequence was typical of the primary structure of aculeacin A acylases (AACs) and penicillin G acylases, with signal peptide-α subunit-spacer-β subunit organization (16, 30). The *Ralstonia* sequence shares substantial identity with AACs from *Deinococcus radiodurans* strain R1, *Actinoplanes utahensis* and a putative acylase from *Pseudomonas aeruginosa*, all of which catalyze deacylation of their substrates. These AAC genes are translated as single precursor polypeptide and then processed to the active

form, which has two subunits. Aculeacin A is an echinocandin-type antifungal antibiotic with a long fatty side chain. Aculeacin A acylases purified from *A. utahensis* catalyze the hydrolysis of the amide bond on the palmitoyl side chain of aculeacin A (29). The primary structure of the protein, as well as enzyme activity analysis with different substrates, discussed below, therefore indicates that *qsbA* encodes an AHL-acylase which cleaves the amide linkage between the acyl side chain and the homoserine lactone moiety of AHLs.

[00028] The presumed α and β subunits of QsbA are located at amino acid positions 36-217 and 233-794, respectively, of SEQ ID NO: 2, with a 15 amino acid spacer between them, as determined by alignment with the peptide sequences from *D. radiodurans* strain R1, *A. utahensis* and *P. Aeruginosa*. See Table II.

Table II. Aligned Amino Acid Sequences of *QsbA* from *Ralstonia sp.* XJ12B (SEQ ID NO: 2), *D. radiodurans* strain R1 acylase (SEQ ID NO: 3), A. *utahensis* acylase (SEQ ID NO: 4) and *P. aeruginosa* acylase (SEQ ID NO: 5).

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R.sp      MMQGF---ALRGTLAMAALAALAGCA-----SSTDGRWGSLSDTGLSAEIRRTGFGIPHIRANDYASLGYGMAYAYAQDN 72
D.rad     MSR-----SPFSSVSLPARLLLGSLL-----LGPLMLGGAASAQTYQVQIQRTAHGIPHIQASDLGGIGYGVGYSYAQDN 70
A.uta     MTSSY---MRLKAAAIAFGVIVATAA-----VPSPAS-GREHDGGYAALIRRASYGVPHITADDFGSLGFGVGYVQAEDN 71
P.aer     MSRPFRPPLCRETTSMGMRTVLTGLAGMLLGSMMPVQADMPRPTGLAADIRWTAYGVPHIRAKDERGLGYGIGYAYARDN 80
          *          : ::    :              .       . *: :..*:*** *.*  .:*:*:.*  *.**

                                   1
R.sp      LCLLADQVVTVNGERSKTFGPEGTVTVSFKPIPNLQSDAFFKGIFDEDGLRAGYAQMSPEARELLRGYIAGFNRYLKDTP 152
D.rad     LCLLADQVMTVRGERSKFLGAEGKTVVGFQPVNNLDSDVFFKTVIEPGRLQAGYRDQ-PQILALMRGYVAGVNRYLRDTP 149
A.uta     ICVIAESVVTANGERSRWFGATGPDDADVRTTSSTQAIDDRVAERLLEGPRDGVRAPCDDVRDQMRGFVAGYNHFLRRTG 151
P.aer     ACLLAEEIVTARGERARYFGSEGKSSAELD---NLPSDIFYAWLNQPEALQAFWQAQTPAVRQLLEGYAAGFNRFLREAD 157
           *::*:.::*..***:: :*. *   . .    .  :

R.sp      PANFP-AACRNAAWVRPLTLGDMMRMGEEKAIQASAGAMLAGIVAAQPPGRTPVAEREIPPQAVDTVALDRELQLRDMPI 231
D.rad     PEQWP-SACRNADWVRPLTELDVMRLGEEKAIQASAGAMVSAITSARPPQ----AGASTAAPRPDLAAFNRQYRFNDLPI 224
A.uta     VHRLTDPACRGKAWVRPLSEIDLWRTSWDSMVRAGSGALLDGIVAATPPT---AAGPASAPEAPDAAAIAAALDGTSAGI 228
P.aer     GKTTS---CLGQPWLRAIATDDLLRLTRRLLVEGGVGQFADALVAAAPPG----AEKVALSGEQAFQVAEQRRQRFRLER 230
             .   * .  *:*.::  *: *      :... * :  .:.:* **     *     .      .
                                                                  1———

R.sp      GSNGWAFGADATANRRGVLLGNPHFPWTTTNRFYQVHLTVPGKLDVMGASIAAFPVVSIGFNKDVAWTHTVSTGRRFTLF 311
D.rad     GSNGWAFGSEATTNGRGLLLGNPHFPWETSNRFYQLHLTLPGQFDVMGASLGGMPVVNIGFNQDVAWTHTVSTDKRFTLA 284
A:uta     GSNAYGLGAQATVNGSGMVLANPHFPWQGAERFYRMHLKVPGRYDVEGAALIGDPIIEIGHNRTVAWSHTVSTARRFVWH 288
P.aer     GSNAIAVGSERSADGKGMLLANPHFPWNGAMRFYQMHLTIPGRLDVMGASLPGLPVVNIGFSRHLAWTHTVDTSSHFTLY 290
          ***. ..*:: :.:  *::*.******  : ***::**.:**: ** **:: . *::.**..: :**:***.*  :*.
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R.sp      ELKLAEGDPTTYLVDGTPHKMTTRTVAFDVKLPDGRLERRTHTFYDTIYGPVLSMPSGGMPWTTQKAYALRDANRNNTRS 391
D.rad     ALTLVPGDPLSYVKDGQQRRLQRRTAVIEVKTANG-PRLHTRTVYFTPEGPLVNLPAAGLTWTPQYAFALRDANRNNTRM 383
A.uta     RLSLVPGDPTSYYVDGRPERMRARTVTVQTGSG-----PVSRTFHDTRYGPVAVVP-GTFDWTPATAYAITDVNAGNNRA 383
P.aer     RLALDPKDPRRYLVDGRSLPLEEKSVAIEVRGADGKLSRVEHKVYQSIYGPLVVWP-GKLDWNRSEAYALRDANLENTRV 390
           * *   **  *  **    :  ::...:.          :..: :  **:   * . : *.   *:*: *.*  *.*

R.sp      VDSWLHIGQARDVAGIRQAIG-NLGIPWVNTIATDRNGRALFADVSTTPDVPAAELQRCAPSPLAGKLFKDAGLVLLDGS 470
D.rad     LATWLGFAGAKSVRDIRASLN-VQGIPWVNTIAADRAGSALYADISSSPNVSAAQQQACTPPPLA-PLFPAAGLAVLDGS 461
A.uta     FDGWLRMGQAKDVRALKAVLDRHQFLPWVNVIAADARGEALYGDHSVVPRVTGALAAACIPAPFQ-PLYASSGQAVLDGS 461
P.aer     LQQWYSINQASDVADLRRRVEALQGIPWVNTLAADEQGNALYMNQSVVPYLKPELIPACAIPQLV-----AEGLPALQGQ 464
          .  *  :  * .*  ::  :     :****.:*:*  * **: : *  * :       *  . :       *   *:*.

R.sp      RGTCNWQVDPASPVPGLVAPARMPVLERDDYVANSNDSSWLTNPAQKLTGFSPVMGSVDVPQRLRTRIGLIEIGRRLAGT 550
D.rad     HSACDWKTDPASRVPGLRAPDKMPVLIRQDFVANSNNSAWLANPAAPQTGLDPLVGEVNAPQSPRTRMGLLEIGRRLSGT 541
A.uta     RSDCALGADPDAAVPGILGPASLPVRFRDDYVTNSNDSHWLASPAAPLEGFPRILGNERTPRSLRTRLGLDQIQQRLAGT 541
P.aer     DSRCAWSRDPAAAQAGITPAAQLPVLLRRDFVQNSNDSAWLTNPASPLQGFSPLVSQE-KPIGPRARYALSRLQGKQP-- 543
           . *    ** :  .*:  .  :**  * *:* ***:* **:.**    *:  ::..

R.sp      DGLPGNRIDLPNLQAMIFSNANLAGQLVLGDLLAACKATPAPDAD------VRDGCAALGQWNRTSNADA-RAAHLFREF 630
D.rad     DGLPGRTFDIPTLQATLLRESNLTGEMYAADAAKLCQS--AGGAE------LQPACNALAAWDRRSSQES-RGAALWREF 619
A.uta     DGLPGKGFTTARLWQVMFGNRMHGAELVRDDLVALCRRQPTATASNGAIVDLTAACTALSRFDERADLDS-RGAHLFTEF 621
P.aer     -------LEAKTLEEMVTANHVFSADQVLPDLLRLCRDN-QGEKS------LARACAALAQWDRGANLDSGSGFVYFQRF 613
                :   *   :  :   .:   *   *:       .     :  .* **. ::. :. ::  .   : .*

R.sp      WMRAKDIAQVHAVEFDPADPVHTPRGLR-MNDATVRTAVFKALKEAVGAVRKAGFALDAPLGTVQAAHAPDGSIALHGGE 702
D.rad     WRRARAIPNVYAVPFDPADPVNTPRGLN-TADPAAQTALLGALREAAAALTAAGIPFDAPLGEVQGVVRGGDFISLPGGA 691
A.uta     LAGG----IRFADTFEVTDPVRTPAPFWNTTDPRVRTALADACNGSPASPSTR------SVGDIHTDSRGERRIPIHGGR 690
P.aer     MQRFAELDGAWKEPFDAQRPLDTPQGIA-LDRPQVATQVRQALADAAAEVEKSGIPDGARWGDLQVSTRGQERIAIPGGD 686
                     *:   *: **  :     . . * :  *   : .            * ::        *.: **

R.sp      EYEGVLNKLQTLPIGPKGLPVYFG--TSYIQTVTFDDQGPVADAILTYGESTDHASPHAFDQMRAYSGKHWNRLPFSEAA 780
D.rad     EFEGVLDKIDFNPLAPGGYRGVVGNASSYIQTVGFTDSGVQAEAVLTYSQSSNPESPYFSDQTRLFSRSEWVKLPFTQPE 771
A.uta     GEAGTFNVITNPLVPGVGYPQVVHG-TSFVMAVELGPHGPSGRQILTYAQSTNPNSPWYADQTVLYSRKGWDTIKYTEAQ 769
P.aer     GHFGVYNAIQS--VRKGDHLEVVGG-TSYIQLVTFPEEGPKARGLLAFSQSSDPRSPHYRDQTELFSRQQWQTLPFSDRQ 763
             *. : :    :   .    .   :*::  * :   *  .  :*::.:*::  **   **   :* . *  : :::

R.sp      IAADPALKVMRLSQ--- 794
D.rad     IEADPTRTVVQLSE--- 785
A.uta     IAADPNLRVYRVAQRGR 786
P.aer     IDADPQLQRLSIRE--- 777
          * ***      : :
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* = identical residues, : = conserved substitutions; . = semi-conserved substitutions; ↑ = post-translational processing sites for signal peptide and subunits; - = spacers.

[00029] The coding region of the *qsbA* gene was amplified by PCR. The amplified PCR products were digested, fused in-frame to the coding sequence of the glutathione S-transferase (GST) gene and expressed in *E. coli*. Protein extracts from the recombinant *E. coli* cells were assayed for the ability to inactivate AHL. Protein from *E. coli* expressing GST alone

served as a control. The results demonstrated that GST-QsbA fusion protein effectively eliminated AHL activity. See Figure 1B.

[00030] The substrate specificity of QsbA was determined by assaying total soluble protein extracted from the recombinant *E. coli* (pGST-QsbA) for inactivation of AHLs using substrates with acyl chains of differing lengths. QsbA was able to completely inactivate 3-oxo group acyl-HSLs having acyl chains of 8, 10 and 12 carbons. QsbA also strongly inactivated methylene group acyl-HSLs having acyl chains of 8 and 10 carbons. QsbA also inactivated the butyl and hexyl esters of N-β-octanoyl-L-homoserine, whereas the AHL-lactonase encoded by *aiiA* was unable to inactivate them. The substrate specificity data indicate that QsbA is an AHL-acylase.

[00031] QsbA and *qsbA* provide new tools for down regulation of AHL-mediated biological activities, such as the expression of virulence genes and biofilm differentiation in pathogenic bacteria, both *in vitro* and *in vivo*. The *qsbA* gene, which encodes the AHL inactivation enzyme (QsbA), or a functional fragment, subunit or substantially homologous variant thereof, may be introduced into a plant genome to produce a genetically modified plant with the ability to quench pathogen quorum-sensing signaling. Transgenic plants expressing an enzyme that inactivates AHLs can exhibit a significantly enhanced resistance to infection by bacterial pathogens, even when bacteria are present in high concentrations.

[00032] Methods of genetic manipulation and transformation of plant cells are well known in the art, as are methods of regenerating fertile, viable transformed plants. In general, any method of cloning the coding region of *qsbA* or a functional fragment or substantially homologous variant thereof into a suitable expression vector may be used. It is convenient to ligate the *qsbA* coding region into a vector, followed by ligation into a plant transformation vector, however those of skill are well aware of alternative methods to achieve the same results. Any suitable plant transformation vector may be used.

The vector contains the *qsbA* gene, or a functional fragment, subunit or substantially homologous variant thereof, so long as expression of the gene results in a QsbA protein or functional fragment, subunit or variant thereof which inactivates AHL.

[00033] A functional promoter preferably controls expression. Many suitable promoters are known in the art, such that a convenient promoter may easily be selected by a skilled artisan depending on the expression system being used. Such selection of a suitable promoter to achieve the desired level of translational expression is considered routine in the art. For example, it is advantageous to optimize *qsbA* expression by modification of codon usage and coupling to a strong promoter such as the double 35S promoter.

[00034] A suitable marker gene, such as kanamycin resistance, green fluorescent protein or any other convenient marker is advantageously used. Variations of the commonly used and well known methods for transforming plants with a gene, are well within the skill of the ordinary artisan in genetic manipulation of plants. Expression constructs may contain a signal sequence to direct secretion of the expressed QsbA protein, or may lack such a sequence, as desired. The plant transformation vectors containing the *qsbA* gene and a marker gene may be used to transform plant cells using *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation is conveniently used to transform plants with the *qsbA* gene, however any suitable method known in the art may be used, depending on the plant being transformed. For example, certain monocotyledonous plants are more efficiently transformed using other methods such as microprojectile bombardment, vacuum filtration or any other method known in the art to introduce and integrate DNA plasmids or fragments into the plant genome. Those of skill in the art are familiar with means to transform gymnosperms, monocots and dicots. All of these methods known in the art are contemplated for use with this invention.

[00035] After selection for transformants carrying the *qsbA* gene, transgenic plants may be regenerated according to known

methods in the art. Plants selected for a marker gene, for example kanamycin resistance, may be assayed, for example by PCR and DNA gel blot to determine how many copies of the *qsbA* gene are present in the plant tissue. Any suitable method known in the art is contemplated for use with the gene of this invention. QsbA enzyme activity may be detected in transgenic plants transformed with the *qsbA* gene by the bioassay method described in Example 2 or by any convenient method.

[00036] By "functional fragment, subunit or substantially homologous variant thereof," when referring to a *qsbA* nucleotide sequence, it is meant any fragment, subunit, variant or homologous sequence of *qsbA* (nucleotides 1234-3618 of SEQ ID NO: 1) which encodes a protein or peptide sequence capable of inactivating N-acyl homoserine lactones. "Substantially homologous variants" of a nucleotide sequence generally are those the complement of which hybridizes with *qsbA* under stringent or highly stringent conditions, for example temperatures of about 30°C to about 50°C, for example 30°C, 35°C, 37°C, 40°C, 45°C or 50°C, and/or salt concentrations of about 200 mM to about 1000 mM NaCl or the equivalent ionic strength, for example 200 mM, 250 mM, 300 mM, 400 mM, 500 mM, 750 mM or 1000 mM. The stringency conditions are dependent on the length of the nucleic acid and the base composition of the nucleic acid and can be determined by techniques well known in the art. Those of skill in the art are familiar with these conditions and ranges which are useful. Generally, a substantially homologous nucleotide sequence is at least about 75% homologous to SEQ ID NO: 1 or a fragment or subunit thereof, preferably at least about 85% homologous, and most preferably 90%, 95% or 99% homologous or more.

[00037] Those of skill in the art are familiar with the degeneracy of the genetic code, and thus are aware that nucleic acid sequences may be less than 100% homologous and yet encode the same protein or peptide sequence. Such variation in any of the sequences, fragments, subunits or substantially homologous variants also are contemplated as part of this invention.

[00038] Peptide and protein sequences which are encompassed by this invention include any sequences encoded by the *qsbA* gene, or any fragment, subunit or substantially homologous variant thereof. Such sequences therefore include any functional protein or peptide which retains the ability to inactivate AHL, including protein and peptide fragments of the complete QsbA protein, such as, for example, the sequences of amino acids 36-217 and 233-794 encoding by SEQ ID NO: 1 and substantially homologous variants thereof. A substantially homologous variant of the QsbA protein includes sequences which are at least about 50% homologous, preferably at least about 60% homologous, and most preferably 70%, 80% or 90% homologous or more. Therefore, a protein which is a substantially homologous varient of QsbA is about 50% to about 99.9% homologous with QsbA. Both conservative and non-conservative amino acid substitutions are contemplated, as well as sequences containing non-traditional or modified amino acids such as those known in the art.

[00039] The term "fragment" is intended to indicate any portion of the nucleotide of SEQ ID NO: 1 or protein/peptide sequence of SEQ ID NO: 2 which is greater than about 300 nucleotide bases or about 100 amino acids, up to one nucleotide or amino acid less than the entire sequence. The term "subunit" is intended to encompass any functional unit of the QbsA protein, such as, for example, amino acids 36-217 or 233-794 of SEQ ID NO: 2.

[00040] A protein or peptide sequence which is considered to inactivate N-acyl homoserine lactones is one which is capable of inactivating at least 55 pmoles N-acyl homoserine lactone (OOHL) per µg protein per minute at 30°C.

[00041] It has been previously demonstrated that quenching bacterial quorum sensing by inactivation of N-acyl homoserine lactone with AHL-lactonase stops bacterial infection (9, 10). The gene and protein described here, which is likely an AHL-acylase, represent a new and effective tool for inactivation of AHL signals and thus control bacterial infection. Similarly,

the gene and protein described here targets AHL quorum-sensing signals that regulate expression of pathogenic genes of many bacterial pathogens at a threshold concentration. This tool is applicable to all plant, animal or human diseases where the expression of pathogenic genes of bacterial pathogens is activated by AHL signals, such as, for example, plant pathogens *Erw. carotovora, Erw. Chrysanthemi, Erw. Stewartii;* human pathogens *P. aeruginosa, B. cepacia;* and animal pathogens X. *nematophilus, P. fluorescens* (1, 3, 6, 12, 17, 19, 22, 23, 24, 26).

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[00042] The following examples are provided to illustrate the invention described herein and should not be construed to limit the appended claims.

EXAMPLES

Example 1. Bacterial Isolation.

[00043] A bacterial biofilm sample was collected from a water treatment system and screened to isolate AHL inactivation bacterial strains. The bacterial mixture was suspended in sterilized water with shaking for 1 hour before spreading onto YEB medium (yeast extract, 5 g/l; casein hydrolysate, 10 g/l; NaCl, 5 g/l; sucrose, 5 g/l; $MgSO_4 7H_2O$, 0.5 g/l and agar, 15 g/l) plates. Individual colonies were restreaked on new plates to ensure purity of the isolates. Bacterial isolates were cultured in LB medium (tryptone, 10 g/L; yeast extract, 5 g/L, and NaCl, 10 g/L; pH7.0) in 1.5-ml Eppendorf™ tubes or 96-well plates at 28°C, with shaking, overnight, and assayed for AHL inactivation activity.

Example 2. AHL Inactivation Bioassay.

[00044] The bacterial culture to be assayed was mixed with an equal volume of fresh medium containing 20 µM N-β-oxooctanoyl-L-homoserine lactone (OOHL), or another AHL, when specified, to form a reaction mixture. The reaction mixture was incubated at 28°C for 4 to 5 hours, followed by 30 minute sterilization under UV light. Plates containing 20 ml MM agar medium (K_2HPO_4, 10.5 g/L; KH_2PO_4, 4.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $FeSO_4$, 4.5 mg/L; $CaCl_2$, 10 mg/L; $MnCl_2$, 2 mg/L; $(NH_4)_2SO_4$, 2.0 g/L; mannitol, 2.0 g/L; pH 7.0) supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, 40 µg/ml) were prepared. The solidified medium was cut, still inside the plate, into separated slices (approximately 1 cm in width). See Figure 1. Five microliters of sterilized reaction mixture was loaded at the top of an MM agar strip, and then AHL indicator cells (*Agrobacterium tumefaciens* strain NT1 (*traR; tra::lacZ749*) (25) 0.7 µl cell suspension with an optical density at 600 nm of 0.4) were spotted at progressively further distances from the loaded samples. Plates were incubated at 28°C for 24 hours. A positive result for AHL inactivation is shown by the absence of blue colonies on the slice. A negative result for AHL inactivation is shown by the presence of blue colonies on the slice. For assay of protein for enzyme activity, total soluble bacterial protein was incubated with 20 µM of OOHL (or other AHL) at 37°C for 1 hour as the reaction mixture.

Example 3. Identification and Cloning the *qsbA* Gene.

[00045] Two bacterial isolates from the biofilm sample with distinct phenotypes, XJ12B and XJ12A, were found to possess the ability to inactivate AHL, with XJ12B showing stronger enzyme activity. The XJ12B isolate was cultured, centrifuged and sonicated. The strongest enzymatic activity was associated with the cell debris fraction rather than the soluble protein and supernatant fractions. These results indicated that the AHL inactivation activity is membrane associated. Sequencing of 16S rRNA was performed to identify the XJ12A and XJ12B isolates.

The 16S rRNA sequences of these isolates showed 97% and 96% identity, respectively, with that of *Ralstonia eutropha*.

[00046] To identify the gene encoding for AHL inactivation, a cosmid library of 1600 clones was constructed in *E. coli* with the genomic DNA of *Ralstonia sp.* strain XJ12B. Genomic DNA from the isolated *Ralstonia sp.* strain XJ12B was partially digested with Sau3A. The resulting DNA fragments were ligated to the dephosphorylated BamH1 site of cosmid vector pLAFR3 (28). The ligated DNA was packed with Gigapack IIIXL Packaging Extract (Stratagene) and transfected into *E. coli* DH5alpha. These *E. coli* transfectants were screened for AHL inactivation activity according to the methods described in Example 2 using OOHL as the substrate. Only a single clone (p13H10) was identified as showing AHL inactivation activity (see Figure 1A, slice 1). To subclone the gene encoding the detected activity, cosmid DNA from the positive clone p13H10 was partially digested with Sau3A and fused into BamH1 digested cloning vector pGEM-3Zf (+). The plasmids were ligated and transformed into *E.coli*, and the *E. coli* were assayed for the ability to inactivate AHL as described in Example 2. Clone p2B10, which contains a 4 kb insert, had AHL inactivation activity (see Figure 1A, slice 2). The TGS™ Template Generation System F-700 (Finnzymes OY) was used to mutate p2B10 plasmid DNA by randomly inserting the artificial Mu transposon, following the manufacturer's instructions. Plasmid clone p2B10, which contains the 4 kb insert containing the *qsbA* gene, was used as a template. Fifteen mutant clones were produced, and none was able to inactive AHL. Bacterial cultures of *E. coli* DH5α containing pMUG3 and pMUC6 are shown as examples in Figure 1A, slices 3 and 4, respectively. Plasmids were subsequently purified for sequencing using primers supplied in the kit.

Example 4. Sequencing and Sequence analysis of the *qsbA* Gene.

[00047] Sequencing was performed according to known methods using ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems). The 4 kb

fragment from clone p2B10 was completely sequenced and is shown in Table I. The sequence contains an open reading frame of 2385 nucleotides with an ATG start codon and a TGA stop codon (SEQ ID NO: 1, nucleotides 1259-3643). Based on the MU transposon mutagenesis data described in Example 3, this open reading frame is the coding region of the AHL inactivation gene, designated as *qsbA*. A putative ribosome binding site (AGGAGA) is located 6 base pairs upstream of the first ATG start codon (underlined in Table I).

[00048] The deduced peptide sequence shows the typical polypeptide primary structure of aculeacin A acylases (AACs) and penicillin G acylases, with signal peptide-α subunit-spacer-β subunit organization (16, 30). There are four additional potential start codons located 3, 36, 189 and 384 downstream from the first ATG. The longest open reading frame encodes 794 amino acids, with a predicted molecular weight of 85373 Daltons. The deduced peptide has 78 strongly basic and strong acid amino acid residues and a predicted isoelectric point of 7.48. The first 20 amino acid residues of the assumed open reading frame appear to be a signal peptide.

[00049] The peptide sequence of *qsbA* deduced from the open reading frame shares 40-52% identity with AACs from *Deinococcus radiodurans* strain R1, *Actinoplanes utahensis* and a putative acylase from *Pseudomonas aeruginosa*, The AACs' catalyze deacylation of their substrates. These AAC genes are translated as single precursor polypeptide and then processed to the active form of two subunits. By alignment with the peptide sequences from *D. radiodurans* strain R1, *A. utahensis* and *P. aeruginosa*, Table II, the presumed α and β subunits are located at amino acid positions 36-217 and 233-794, respectively, with a 15 amino acid spacer between them. *QsbA* shares less than 28% homology with penicillin G acylase (20) and cephalosporin acylase (21). See Table II. The amino acid sequence alignment in Table II was analyzed by the Clustal W program available from the European Bioinformatics Institute website (http://www.ebi.ac.uk).

Example 5. Expression of the *QsbA* Gene.

[00050] The coding region of the *qsbA* gene was amplified by PCR using a forward primer 5'-CGTGGATCCATGATGCAGGATTCGCCGCTGCGC-3' (SEQ ID NO: 6) and a reverse primer 5'- CGCGAATTCACCGGCAGCCCTCATGCGACAAC-3' (SEQ ID NO: 7) containing BamH1 and EcoR1 restriction sites, respectively. The amplified PCR products were digested using the above restriction enzymes and fused in-frame to the coding sequence of the glutathione S-transferase (GST) gene under the control of the isopropyl β-D-thiogalactopyranoside (IPTG) inducible lac promotor in vector pGEX-2T (Amersham Pharmacia) to generate construct pGST-QsbA. pGST-QsbA was transformed into *E. coli* and expressed.

[00051] Total soluble protein was extracted from the recombinant *E. coli* cells harboring the GST-QsbA-encoding fusion construct according to methods known in the art, based on the methods described in Dong et al. (9), and assayed for AHL inactivation. The total soluble protein from *E. coli* containing GST vector only was used as a control. For the bioassay, 50 µl of the soluble protein preparation (20 µg/µl) was added to the same volume of 40 µM AHL, *e.g.*, OOHL. After a 1 hour incubation at 37°C, the reaction mixture was assayed as described in Example 2. Representative data, shown in Figure 1B, slice 1, indicate that the soluble GST-QsbA fusion protein effectively eliminated OOHL activity.

Example 6. Characterization of the Substrate Spectrum of GST-QsbA Fusion Protein Expressed in *E. coli*.

[00052] To determinate the substrate spectrum of QsbA, total soluble protein extracted from the recombinant *E. coli* (pGST-QsbA) was assayed for inactivation of AHLs with acyl chains of differing lengths according to the methods of Example 2. The following AHLs were synthesized according to known methods as described by Zhang et al. (31): (1) N-octanoyl-L-homoserine lactone (C8HSL, OOHL); (2) N-decanoyl-L-homoserine lactone (C1DHSL, DHL); (3) N-β-oxohexanoyl-L-homoserine lactone (3-oxo-

C6HSL, OHHL); (4) N-β-oxohexanoyl-L-homoserine lactone (3-oxo-C12HSL, OdDHL); (5) N-β-oxohexanoyl-L-homoserine lactone (3-oxo-C8HSL, OOHL). The butyl and hexyl esters of N-β-oxohexanoyl-L-homoserine were prepared by esterification of N-β-oxohexanoyl-L-homoserine lactone with 1-butanol and 1-hexanol respectively, in the presence of small amount of Dowex 50H+ resin (Aldrich). The reaction was conducted at 60°C for 2 hours and the products were purified by silica column chromatography.

[00053] QsbA completely inactivated OOHL, N-β-oxodecanoyl-L-homoserine (ODHL) and N-β-oxododecanoyl-L-homoserine (OdDHL), which have acyl chains of 8, 10 and 12 carbons, respectively, at the concentrations tested (data not shown). QsbA also strongly inactivated N-β-octanoyl-L-homoserine(OHL) and N-β-decanoyl-L-homoserine(DHL), which have acyl chains of 8 and 10 carbons, respectively (data not shown). However, under the same reaction conditions, QsbA had less inactivating activity for N-β-oxohexanoyl-L-homoserine (OHHL), which has an acyl chain of 6 carbons (data not shown). The total soluble protein extract from control *E.coli* (pGST) did not show any activity against AHLs (data not shown).

[00054] QsbA also completely inactivated the butyl and hexyl esters of N-β-octanoyl-L-homoserine (data not shown). These two esters of N-β-octanoyl-L-homoserine showed comparable induction activity with OOHL when assayed with the AHL reporter strain *A. tumefaciens* NT1 (*traR; tra::lacZ749*) (data not shown). AHL-lactonase (encoded by *aiiA*) did not inactivate these substrates. These substrate specificity data are consistent with identification of QsbA as an AHL-acylase.

Example 7. Purification of AHL-acylase encoded by the *qsbA* gene

[00055] The GST-[AHL-acylase] fusion protein was purified using a glutathione Sepharose 4B affinity column following the manufacturer's instructions (Pharmacia). AHL-acylase was cleaved by digestion with thrombin (Sigma). Protein concentration was determined by measuring OD_{280}.

[00056] The purified AHL-acylase was incubated with OOHL for 20 minutes and the relative enzyme activity was measured by determining the residual OOHL in the reaction mixture, which contained 8 µM OOHL and about 0.6 µg AHL-acylase in a total reaction volume of 50 ml 1x PBS buffer. The reactions were stopped by addition of 1% SDS before loading on the assay plate. Determination of the OOHL activity was carried out in quadruplicate. AHL-acylase degraded OOHL in a range of temperatures from 22-42°C at pH 7.0. See Figure 2. The optimal temperature for enzyme activity was found to be approximately 28°C. Reaction temperature higher than 42°C decreased enzyme activity sharply. The optimal pH for enzyme activity also was determined. The AHL-acylase has a relatively narrow optimal pH range from pH 6.5 to 7.5. See Figure 2. The time course of OOHL inactivation by the purified AHL-acylase was determined at 30°C. See Figure 3. After 10 minutes, more than 82% OOHL had been degraded; the reaction rate was estimated to be about 55 pmols per µg AHL-acylase per minute.